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Inhibition of eNOS phosphorylation mediates endothelial dysfunction in renal failure: new effect of asymmetric dimethylarginine

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Patients with chronic kidney disease have elevated circulating asymmetric dimethylarginine (ADMA). Recent studies have suggested that ADMA impairs endothelial nitric oxide synthase (eNOS) by effects other than competition with the substrate L-arginine. Here, we sought to identify the molecular mechanism by which increased ADMA causes endothelial dysfunction in a chronic kidney disease model. In wild-type mice with remnant kidney disease, blood urea nitrogen, serum creatinine, and ADMA were increased by 2.5-, 2-, and 1.2-fold, respectively, without any change in blood pressure. Nephrectomy reduced endothelium-dependent relaxation and eNOS phosphorylation at Ser1177 in isolated aortic rings. In transgenic mice overexpressing dimethylarginine dimethylaminohydrolase-1, the enzyme that metabolizes ADMA, circulating ADMA was not increased by nephrectomy and was decreased to half that of wild-type mice. These mice did not exhibit the nephrectomy-induced inhibition of both endothelium-dependent relaxation and eNOS phosphorylation. In cultured human endothelial cells, agonist-induced eNOS phosphorylation and nitric oxide production were decreased by ADMA at concentrations less than that of L-arginine in the media. Thus, elevated circulating ADMA may be a cause, not an epiphenomenon, of endothelial dysfunction in chronic kidney disease. This effect may be attributable to inhibition of eNOS phosphorylation.

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KEYWORDS: asymmetric dimethylarginine; chronic kidney disease; endothelial dysfunction; nitric oxide synthase; signal transduction

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The prevalence of chronic kidney disease (CKD) is increasing worldwide.[1,2] Approximately 50% of the end-stage renal disease patients die from cardiovascular causes[3–5] and their cardiovascular mortality is 500-fold greater compared with that of age-matched controls with normal renal function.[5] The Framingham Heart Study revealed that mild renal failure was associated with increased prevalence of death and cardiovascular events even in the general population.[6] Endothelial dysfunction was documented from the early stage of renal failure,[7] suggesting that endothelial dysfunction is one of the initial mechanisms that lead to cardiovascular complications in CKD patients.

Asymmetric dimethylarginine (ADMA) is generated during the process of protein turnover and is actively degraded by the intracellular enzyme, dimethylarginine dimethylaminohydrolase (DDAH).[8–13] ADMA is an endogenous inhibitor of all types of nitric oxide synthases (NOSs).[14,15] It has long been thought that the NOS inhibition by ADMA is attributable to its competitive inhibition as an L-arginine analog.[10,16] However, there is increasing evidence that ADMA may have additional effects that are independent of the competitive inhibition of NOS although the precise mechanisms are unknown.[17–19]

We have shown that circulating ADMA levels are correlated with the thickness of the carotid artery in a general healthy population.[20] Thereafter, ADMA has been increasingly recognized as a putative biomarker in cardiovascular diseases.[21] It was reported that circulating ADMA levels were elevated in CKD patients.[16,22,23] Reduced bioavailability of nitric oxide (NO) has been documented in CKD patients, concurrently with endothelial dysfunction.[24–26] However, it remains undetermined whether the elevation of circulating ADMA level is a cause or an epiphenomenon of endothelial damages in patients or animal models of CKD.

The aims of this study were to examine whether increased circulating ADMA causes endothelial dysfunction in a mouse model of CKD and, if so, to investigate the molecular mechanism. To address the contribution of circulating ADMA to endothelial dysfunction in CKD, we created 5/6

nephrectomized (Nx) models in DDAH-1 overexpressed mice having reduced circulating ADMA levels. The effects of ADMA on endothelial NOS (eNOS) activity were investigated in cultured human umbilical vein endothelial cells (HUVECs).

RESULTS

5/6 Nx causes renal failure without hypertension

Nx increased blood urea nitrogen (BUN) and serum creatinine levels in wild-type (WT) mice. The elevations of BUN and serum creatinine in human DDAH-1–transgenic (TG) mice receiving Nx (TG + CKD) were similar to those in WT mice receiving Nx (WT + CKD) 4 weeks after the operation (Figure 1a and b). There were no differences in systolic blood pressure and heart rate among the four groups (Figure 1c and d). We did not observe apparent morphological and histological changes in the heart and aorta in WT + CKD, TG + sham, and TG + CKD mice (data not shown).

Serum ADMA levels

In WT + CKD mice, serum ADMA levels were increased by 20% compared with those in WT + sham mice (Figure 1e). TG + sham mice showed a 50% reduction in serum ADMA levels compared with WT + sham mice. In TG mice, Nx did not increase the ADMA levels.

Endothelium-dependent relaxation of aortic rings

In WT + sham mice, acetylcholine (Ach) induced a dose-dependent relaxation of the aortic rings precontracted with phenylephrine (Figure 2 and Supplementary Table S1 online). The endothelium-dependent relaxation was significantly impaired in isolated aortic rings obtained from WT + CKD mice. In TG + sham mice, the endothelium-dependent relaxation was similar to that in WT + sham mice. The Nx-induced endothelial dysfunction was prevented in TG mice. These results were confirmed by the area under the curve analysis (Table 1). The half-maximal inhibitory concentration (IC_{50}) of Ach was significantly greater in WT + CKD mice compared with WT + sham mice, suggesting that ADMA reduced the sensitivity for Ach after Nx in WT mice. However, the IC_{50} levels in TG + sham and TG + CKD mice did not differ from that in WT + sham mice (Table 1).

Vascular eNOS expression and phosphorylation in CKD mice

The Ser1177 residue of eNOS is a key phosphorylation site that positively regulates eNOS enzyme activity independently of intracellular calcium concentrations.[27] We investigated the eNOS expression and phosphorylation levels in the aorta of WT and TG mice with or without Nx (Figure 3a and b). Nx did not affect eNOS protein expression levels in WT mice. However, eNOS phosphorylation at Ser1177 was attenuated by Nx, suggesting the inhibition of eNOS activity in WT + CKD mice. In TG + sham mice, the eNOS expression and phosphorylation levels were similar to those in WT + sham mice. The reduction in eNOS phosphorylation observed in WT + CKD mice was abolished in TG + CKD mice. In addition, we examined urinary nitrate/nitrite (NOx) excretion in 24 h as an indicator of NO production *in vivo* (Figure 3c). Urinary NOx excretion was reduced by 66% in WT + CKD mice compared with WT + sham mice. In TG + sham mice, urinary NOx excretion was much higher than that in WT + sham mice. The NOx excretion was significantly reduced in TG + CKD mice compared with TG + sham mice. But, the NOx levels in TG + CKD mice were similar to the levels in WT + sham mice and significantly higher than those in WT + CKD mice.

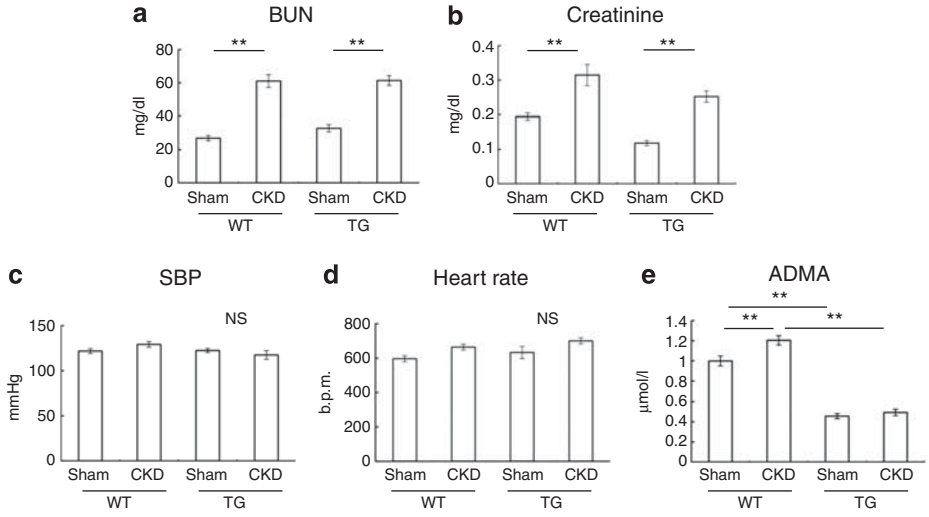

Figure 1 | Nephrectomy (Nx) causes moderate renal failure and increases circulating asymmetric dimethylarginine (ADMA). The effects of Nx on blood urea nitrogen (BUN) (**a**), serum creatinine (**b**), systolic blood pressure (SBP) (**c**), heart rate (**d**), and serum ADMA (**e**) at 4 weeks after Nx or sham operation in wild-type (WT) mice and dimethylarginine dimethylaminohydrolase-1 transgenic (TG) mice. WT mice receiving sham operation (WT + sham), $n = 9$; WT mice receiving Nx (WT + CKD), $n = 9$; TG mice receiving sham operation (TG + sham), $n = 6$; TG mice receiving Nx (TG + CKD), $n = 6$. Values are mean ± s.e.m. $^{**}P < 0.01$. b.p.m., beats per minute; CKD, chronic kidney disease; NS, not significant.

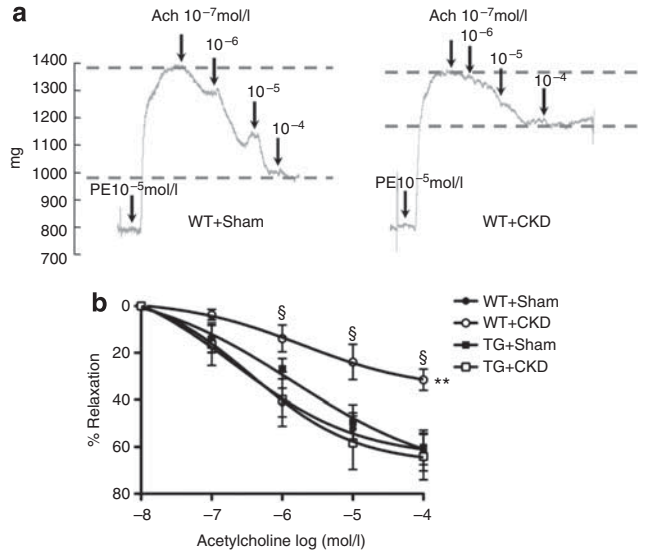

Figure 2 | Endothelium-mediated relaxation is impaired in the aorta of wild-type (WT) + CKD mice, but not in transgenic (TG) + CKD mice. (**a**) Representative traces showing the acetylcholine (Ach)-induced relaxation in phenylephrine (PE)-pre-treated rings of the descending aorta obtained from WT + sham (left) and WT + CKD (right) mice. (**b**) Pooled data of the Ach-induced relaxation in WT + sham ($n = 7$), WT + CKD ($n = 4$), TG + sham ($n = 5$), and TG + CKD ($n = 4$). Values are mean ± s.e.m. $^{**}P < 0.01$ vs. WT + sham mice as a result of two-way analysis of variance. $^{\S}P < 0.05$ vs. WT + sham mice as a result of Tukey–Kramer's *post-hoc* analysis. CKD, chronic kidney disease.

Table 1 | Effects of CKD on IC_{50} and AUC of acetylcholine-induced relaxation of the aortic rings of WT and TG mice

		Acetylcholine	
Group	*n*	Log IC_{50} (mol/l)	AUC (arbitrary unit)
WT+sham	9	−6.67 ± 0.62	139 ± 21
WT+CKD	4	−5.72 ± 0.78*	49 ± 13*
TG+sham	5	−5.85 ± 0.65	121 ± 16
TG+CKD	4	−6.44 ± 0.67	146 ± 36

Abbreviations: AUC, area under the curve; CKD, chronic kidney disease 5/6 nephrectomized mouse; IC_{50}, half-maximal (50%) inhibitory concentration; Sham, sham-operated control mouse; TG, dimethylarginine dimethylaminohydrolase-1 transgenic mouse; WT, wild-type mouse.
*$P < 0.05$ vs. WT + sham.
Data are expressed as mean ± s.e.m.

Effects of ADMA on endothelial-dependent relaxation

To determine whether ADMA would directly impair endothelial function, we examined the effects of exogenous ADMA treatment on the endothelium-dependent relaxation of the aorta in WT mice (Figure 4). The endothelium-dependent relaxation was dose-dependently inhibited by pre-treatment with 1×10^{-6}–1×10^{-4} mol/l ADMA, although the inhibition was not statistically significant for 10^{-6} mol/l ADMA (Figure 4b and Table 2). The IC_{50} of Ach was significantly increased by 1×10^{-4} mol/l ADMA pre-treatment compared with 0 mol/l ADMA (Table 2).

Effects of ADMA on eNOS function in HUVECs

To further determine the molecular mechanisms of the endothelial dysfunction by ADMA, we examined the effects of ADMA on eNOS phosphorylation using cultured HUVECs (Figure 5a and b). In HUVECs cultured in the media containing 5×10^{-4} mol/l L-arginine, ADMA (1×10^{-6} and 1×10^{-4} mol/l) did not affect the baseline eNOS phosphorylation at Ser1177. However, ADMA dose-dependently inhibited the vascular endothelial growth factor-induced eNOS phosphorylation. Next, we examined the effects of ADMA on the phosphorylation of extracellular signal-related protein kinase (ERK) and Akt, putative upstream regulatory molecules of eNOS phosphorylation. ADMA dose-dependently inhibited the vascular endothelial growth factor-induced ERK1/2 phosphorylation, without changing the baseline phosphorylation. In contrast, ADMA did not affect Akt phosphorylation at Ser473. Moreover, we confirmed the effects of ADMA on NO production in HUVECs. ADMA inhibited the vascular endothelial growth factor-stimulated NO release into the conditioned media (Figure 5c).

DISCUSSION

The salient findings of this study are as follows: (1) Nx induced moderate renal failure to a similar extent in WT and DDAH-1–TG mice without elevating blood pressure; (2) Nx impaired endothelium-dependent relaxation of isolated aortic rings in WT mice with the elevation of circulating ADMA levels, but not in TG mice having reduced ADMA levels; (3) Nx attenuated eNOS phosphorylation at Ser1177, an indicator of eNOS activity, in the aorta of WT mice, but not in TG mice; (4) ADMA treatment suppressed the agonist-induced eNOS phosphorylation and NO production in HUVECs cultured under the L-arginine-rich condition.

As shown in Figure 1, a mouse with Nx had 2.5- and 2-fold increases in BUN and creatinine, respectively, without blood pressure elevation, suggesting that it is a CKD model representing moderate renal failure without hypertension. This study was performed in young mice, and the observation period was rather short because we focused on investigating the relationship between elevated circulating ADMA and endothelial function in the CKD model before cardiovascular damages and remodeling had developed. TG mice showed a 50% reduction in baseline ADMA levels (Figure 1e). This finding is consistent with that of previous reports.[28,29] Although circulating ADMA levels were significantly increased by Nx in WT mice, Nx did not change the ADMA levels in TG mice, probably due to augmented ADMA degradation by overexpressed DDAH-1. Thus, TG + CKD mice were used as a model animal having renal failure without elevated circulating ADMA levels. It is noteworthy that the Nx-induced impairment of endothelium-dependent relaxation was prevented in TG + CKD mice (Figure 2 and Supplementary Table S1 online). As the blood pressure level and the extent of renal failure were similar in WT + CKD and TG + CKD mice (Figure 1), it is suggested that the elevation of circulating ADMA levels has a causal relation to the Nx-induced endothelial dysfunction in WT mice (Figures 2 and 4). To the best of our knowledge, this is the first

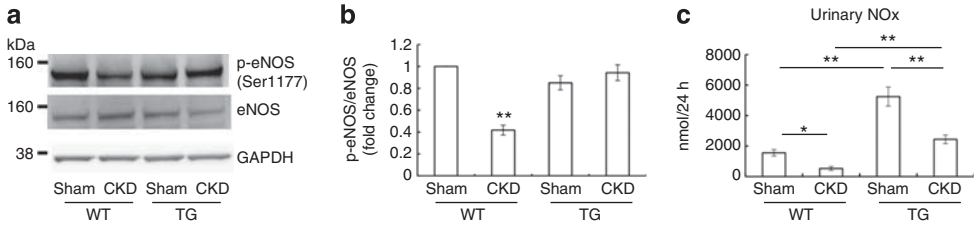

Figure 3 | Vascular endothelial nitric oxide synthase (eNOS) activity is impaired in the aorta of wild-type (WT) + CKD mice, not transgenic (TG) + CKD mice. Representative immunoblots (**a**) and the pooled data (**b**) demonstrating the effects of nephrectomy (Nx) on the expression levels of eNOS, phosphorylated eNOS (p-eNOS), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the aorta of WT + sham ($n = 9$), WT + CKD ($n = 9$), TG + sham ($n = 4$), and TG + CKD ($n = 4$). The results of densitometric analysis were presented as a fold change compared with WT + sham. $^{**}P < 0.01$ vs. WT + sham, TG + sham, and TG + CKD. (**c**) Urinary excretion of total nitrate and nitrite (NOx) in WT + sham ($n = 10$), WT + CKD ($n = 10$), TG + sham ($n = 6$), and TG + CKD ($n = 6$). $^{*}P < 0.05$, $^{**}P < 0.01$. Values are mean ± s.e.m. CKD, chronic kidney disease.

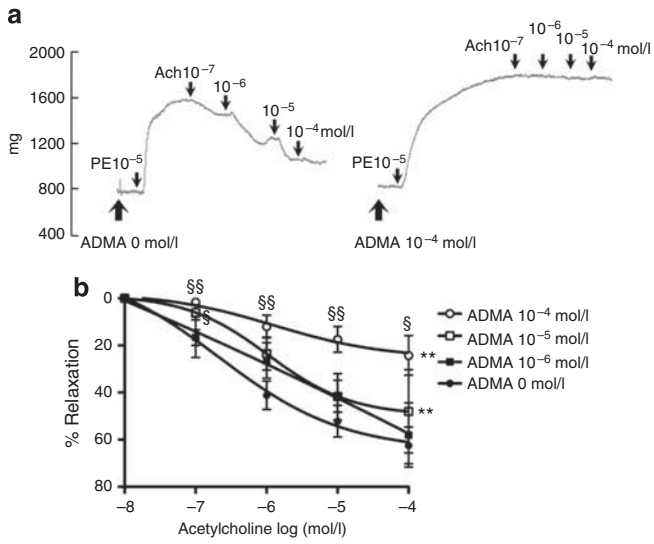

Figure 4 | Asymmetric dimethylarginine (ADMA) impairs endothelium-mediated relaxation in the aorta. Representative traces (**a**) and pooled data (**b**) showing the effects of 1×10^{-6}–1×10^{-4} mol/l exogenous ADMA on the acetylcholine (Ach)-induced relaxation of the phenylephrine (PE)-precontracted aortic rings obtained from wild-type (WT) + sham mice. After 10-min pre-treatment with 0 mol/l (left) or 1×10^{-4} mol/l ADMA (right), 1×10^{-5} mol/l PE was applied to the strips. $^{**}P < 0.01$ vs. ADMA 0 mol/l as a result of two-way analysis of variance. $^{\S}P < 0.05$, $^{\S\S}P < 0.01$ vs. ADMA 0 mol/l as a result of Tukey–Kramer's *post-hoc* analysis.

Table 2 | Effects of ADMA on IC_{50} and AUC of acetylcholine-induced relaxation of the aortic rings of WT mice

		Acetylcholine	
Group	*n*	Log IC_{50} (mol/l)	AUC (arbitrary unit)
ADMA 10^{-4} mol/l	9	−5.53 ± 0.31**	31 ± 9*
ADMA 10^{-5} mol/l	6	−6.36 ± 0.27	73 ± 24*
ADMA 10^{-6} mol/l	6	−6.26 ± 0.25	100 ± 32
ADMA 0 mol/l	9	−6.64 ± 0.13	125 ± 23

Abbreviations: ADMA, asymmetric dimethylarginine; AUC, area under the curve; IC_{50}, half-maximal (50%) inhibitory concentration; WT, wild type.
$^{*}P < 0.05$, $^{**}P < 0.01$ vs. ADMA 0 mol/l.
Data are expressed as mean ± s.e.m.

demonstration that elevated circulating ADMA may be a cause, but not an epiphenomenon, of endothelial dysfunction in CKD.

Another important novel finding of this study is that reduction of eNOS phosphorylation at Ser1177 was associated with endothelial dysfunction in the aorta of WT + CKD mice, but not in TG + CKD mice (Figure 3). Phosphorylation levels at Ser1177 determine eNOS enzyme activity.[27] Indeed, the urine NOx excretion, an indicator of NO production *in vivo*, was significantly higher in TG + CKD mice than in WT + CKD mice, suggesting eNOS phosphorylation levels in the aorta are functionally associated with NO production in the mice. Thus, it is suggested that the reduced eNOS phosphorylation may be involved in the mechanism accounting for the endothelial dysfunction in WT + CKD model. The precise mechanism linking ADMA to inhibition of eNOS phosphorylation remains unknown in this CKD model. However, the experiments using HUVECs provided an insight into the possible mechanism (Figure 5). It is interesting to note that ADMA prevented the agonist-induced eNOS phosphorylation and NO production in HUVECs. Moreover, the phosphorylation of ERK, one of the putative major kinases for eNOS phosphorylation, was also inhibited by ADMA. Thus, it is possible that the inhibition of ERK–eNOS pathway would be a mechanism whereby ADMA impairs eNOS function. We used the culture media containing L-arginine, the eNOS substrate, at 5×10^{-4} mol/l, which was a much higher concentration of ADMA that was applied to HUVECs (1×10^{-6} and 1×10^{-4} mol/l). In this study, it is less likely that competitive inhibition was the reason for the observed effects of ADMA on NO generation. Rather, the inhibition of the ERK–eNOS phosphorylation may be a novel mechanism of eNOS inhibition by ADMA (Figure 6). These findings may support the notion that ADMA has additional actions other than competitive inhibition of eNOS.[17–19] It is noteworthy that the inhibitory effect of ADMA on eNOS phosphorylation in HUVECs was observed at 1×10^{-6} mol/l, which corresponds to the serum levels of ADMA in CKD mice (Figure 1). Taken together, it is possible that the inhibition of the ERK–eNOS phosphorylation by elevated circulating ADMA levels would impair the endothelium-dependent relaxation in CKD mice.

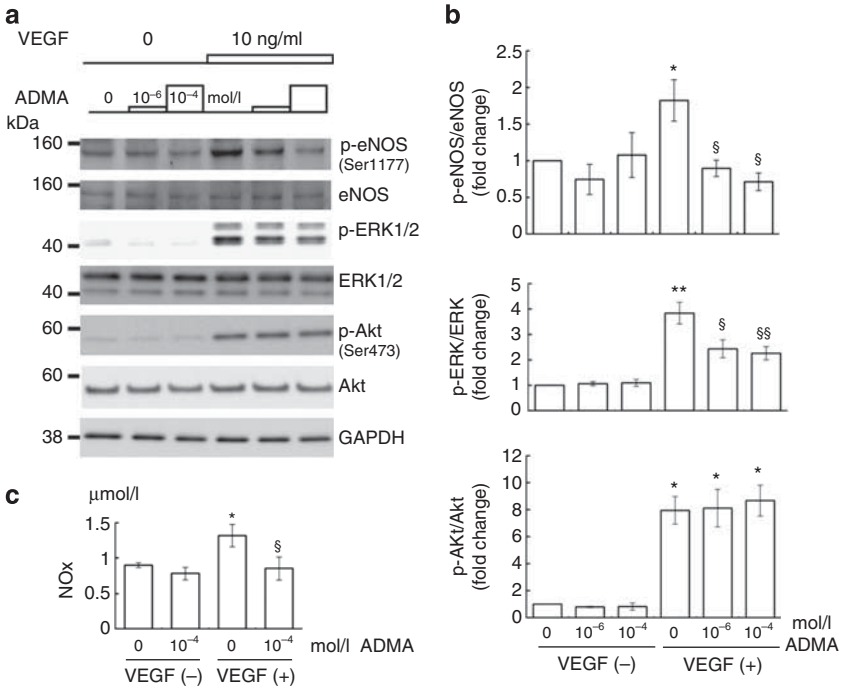

Figure 5 | Asymmetric dimethylarginine (ADMA) decreased the agonist-induced phosphorylation of endothelial nitric oxide synthase (eNOS) and the upstream kinases. Representative immunoblots (**a**) and the pooled data (**b**) showing the effects of ADMA on the basal and vascular endothelial growth factor (VEGF)-stimulated phosphorylation levels of eNOS, extracellular signal-related protein kinase (ERK), and Akt in human umbilical vein endothelial cells (HUVECs). The results of densitometric analysis were presented as a fold change compared with untreated cells. (**c**) Pooled data showing the effects of ADMA on the nitrate and nitrite (NOx) concentrations in the conditioned media of HUVECs with or without VEGF treatment. After 6-h pre-treatment with ADMA, VEGF was administered to HUVECs. Values are mean ± s.e.m. $n = 6$ per each group, $^{*}P < 0.05$, $^{**}P < 0.01$ vs. VEGF(−)/ADMA 0 mol/l. $^{\S}P < 0.05$, $^{\S\S}P < 0.01$ vs. VEGF(+)/ADMA 0 mol/l. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; p-eNOS, phosphorylated eNOS.

This study has several limitations. First, we used a tail-cuff sphygmomanometer to noninvasively measure blood pressure without anesthesia. Thus, we do not deny the possibility that we may have missed subtle blood pressure differences. Second, we used a human DDAH-1 TG expression construct using a human β-actin promoter in this study, because the endothelium-specific TG mice were not available at present. Thus, we verified the overexpression of DDAH-1 in the endothelial cells as well as smooth muscle cells of the aorta in TG mice (Supplementary Figure S1 online). Third, 10^{-6} mol/l ADMA reduced eNOS phosphorylation in the agonist-stimulated HUVECs (Figure 5), whereas Ach-induced vasorelaxation of the aortic rings was not significantly inhibited by 10^{-6} mol/l ADMA (Figure 4). It is difficult to simply compare the results of the HUVEC experiments and the isometric tension study because there were many differences in the experimental conditions. Moreover, we did not know the intracellular concentrations of ADMA when the same dose of ADMA was applied to the HUVECs and the aortic rings. Finally, *in vivo* WT + CKD mice had an attenuated basal eNOS phosphorylation because of CKD-induced elevation of ADMA (Figure 3a), whereas *in vitro* exogenous administration of ADMA did not affect basal eNOS phosphorylation (Figure 5a and b). There may be several reasons for this difference. One possibility is that there may be currently unknown stimuli that induce basal eNOS phosphorylation in the aorta of mice. Another may be that in the HUVEC experiments, the effects of ADMA on basal eNOS phosphorylation were not detected simply because the basal phosphorylation level was low and/or because the changes by ADMA were too small to be detected.

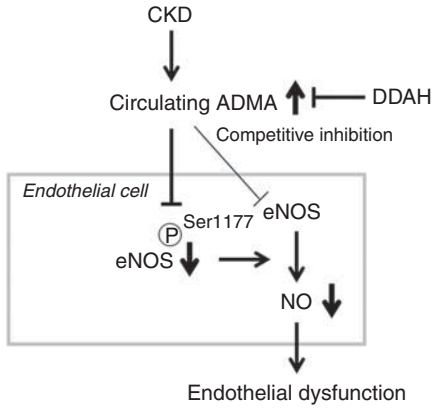

Figure 6 | Proposed mechanism for endothelial dysfunction in chronic kidney disease (CKD). The increase in circulating asymmetric dimethylarginine (ADMA) causes endothelial dysfunction in CKD by reducing vascular endothelial nitric oxide synthase (eNOS) phosphorylation. Inhibition of the eNOS signaling pathway may be an additional mechanism accounting for the eNOS inhibition by ADMA in CKD, independently of competitive inhibition. DDAH, dimethylarginine dimethylaminohydrolase; NO, nitric oxide.

In conclusion, increased circulating ADMA level caused endothelial dysfunction in CKD mice. It was suggested that the effect might be mediated by the inhibition of eNOS phosphorylation, a novel mechanism of eNOS inhibition by ADMA.

MATERIALS AND METHODS

The study protocol was reviewed and approved by the Animal Care and Treatment Committee of Kurume University. C57BL/6J mice, WT mice, were purchased from Charles River Laboratories (Yokohama, Japan). Human DDAH-1–TG mice in C57BL/6J background were purchased from Jackson Laboratory (Bar Harbor, ME) and genotyped as previously described.[28–30] A human DDAH-1 TG expression construct was prepared using human DDAH-1 complementary DNA, a human β-actin promoter, and RNA processing signals from SV40 derived from a modified human agouti expression vector, as described.[28] Mice were housed under standard conditions of humidity, room temperature, and a 12-h light/12-h dark cycle with plenty of chow and water. Male mice were used for all experiments. HUVECs were purchased from Lonza (Basel, Switzerland).

A rabbit monoclonal antibody against human eNOS, a rabbit polyclonal antibody against human phosphorylated eNOS at Ser1177, a rabbit monoclonal antibody against mouse Akt, a rabbit monoclonal antibody against human phosphorylated Akt at Ser473, a rabbit polyclonal antibody against rat ERK, and a rabbit monoclonal antibody against human phosphorylated ERK at Thr202/Tyr204 were purchased from Cell Signaling Technology (Danvers, MA), and a mouse monoclonal antibody against rabbit glyceraldehyde-3-phosphate dehydrogenase was from Millipore (Billerica, MA).

Animal model

Experiments included the following four groups: WT mice with sham operation (WT + sham mice, $n = 30$); WT mice receiving Nx (WT + CKD mice, $n = 27$); TG mice with sham operation (TG + sham mice, $n = 24$); and TG mice receiving Nx (TG + CKD mice, $n = 20$).

CKD mice were created by performing Nx in WT and TG mice as previously described.[31] Briefly, mice were anesthetized with 1.5% isoflurane by inhalation. 5/6 Nx was established by surgical resection of the upper and lower thirds of the left kidney at 10–12 weeks followed by right Nx 1 week later. Four weeks after the establishment of Nx, blood pressure and heart rate were measured using a tail-cuff sphygmomanometer (MK-2000ST; Muromachi, Tokyo, Japan). Briefly, mice were acclimated to a holding chamber by daily exposure for 1 week. Once acclimated, systolic blood pressure was measured by a tail-cuff sphygmomanometer. A total of 10 consecutive readings of systolic blood pressure were recorded and averaged. The interobserver and intraobserver variabilities were below 5%. One day after measuring the blood pressure and heart rate, mice were killed with an overdose of pentobarbital (100 mg/kg intraperitoneally). Blood was collected from the right appendage, and then perfused with ice-cold saline for 5 min. The aorta was immediately removed and subjected to isometric tension study or immunoblotting analysis. For urine collection, mice were housed in metabolic cages for 24 h before killing.

Blood and urine chemistry

Serum ADMA and urine NOx (oxidized derivatives of NO) were measured using high-performance liquid chromatography at a commercially available laboratory (SRL, Tokyo, Japan).[32,33] BUN and serum creatinine were measured with commercially available kits (Denka Seiken, Tokyo, Japan and Alfresa Pharma, Osaka, Japan, respectively).

Isometric tension study

Aortic rings obtained from the descending thoracic aorta of WT mice and TG mice were mounted on a wire myograph (ADInstruments Pty, Bella Vista, Australia) for the endothelial function assay. After 60-min equilibration in an organ bath containing Krebs' solution aerated with 95% CO_2–5% O_2 (37 °C), aortic rings were pre-constricted with 1×10^{-5} mol/l phenylephrine. Then, an incremental dose of Ach (1×10^{-7}–1×10^{-4} mol/l) was applied at 10-min intervals. To determine the effect of exogenous ADMA in the isometric tension study, Ach-induced (Sigma-Aldrich, St Louis, MO) relaxation was assessed in the presence of ADMA (Sigma-Aldrich) in WT mice ($n = 18$). According to a previous study,[34] 1×10^{-6}–1×10^{-4} mol/l ADMA was applied to the aortic rings. Two-way analysis of variance followed by *post-hoc* analysis and the area under the curve analysis was performed to compare the differences among four groups. IC_{50} of Ach was calculated for evaluating the sensitivity to Ach.

Immunoblotting

The aorta was immediately removed and snap-frozen in liquid N_2. Frozen samples were homogenized in lysis buffer containing protease inhibitor cocktail by using FastPrep homogenizer (Thermo Savant, Holbrook, NY) and stored at −80 °C until use. The aliquot of tissue homogenate or cell lysate was separated on 4–12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels and subjected to immunoblotting using a primary antibody (1:100 dilution) and a peroxidase-conjugated anti-rabbit or anti-mouse secondary antibody (1:5000 dilution). The immunoreactive bands were detected using ECL western blotting reagents (Thermo Fisher Scientific, Waltham, MA). The intensity of immunoreactive bands was quantified by densitometry (MultiGauge software, Fujifilm Holdings, Tokyo, Japan). eNOS and phospho-eNOS, ERK and phospho-ERK, or Akt and phospho-Akt were detected on the same gel following re-probing of membranes. The signal intensities of the phosphorylated eNOS, ERK, and Akt were normalized by the intensities of the total eNOS, ERK, and Akt in each membrane, respectively.

Cell culture

HUVECs (1×10^5 cells/ml) were cultured in EGM-2 medium (Sanko Junyaku, Tokyo, Japan) supplemented with 2% fetal bovine serum. EGM-2 medium contains L-arginine of 5×10^{-4} mol/l. Passages 5–10 were used for the experiments. After the HUVECs were growth-arrested in serum-free medium for 24 h, cells were incubated with 1×10^{-6} or 1×10^{-4} mol/l ADMA or the vehicle for 6 h. Ten minutes after 10 ng/ml recombinant vascular endothelial growth factor (R&D Systems, Minneapolis, MN) or the vehicle was applied to HUVECs, the reaction was terminated by aspirating the medium. NO production was estimated by measuring levels of NOx in the conditioned medium with an assay kit (Dojindo Laboratories, Kumamoto, Japan).[35] After three washes with ice-cold phosphate-buffered saline on ice, cells were homogenized in lysis buffer containing protease inhibitor cocktail.

Statistical analysis

Results are shown as mean ± s.e.m. Area under the curve and IC_{50} values were calculated by GraphPad Prism (GraphPad, San Diego, CA)

computer software using non-linear sigmoid curve fitting. Intergroup differences were assessed by the Mann–Whitney *U*-test or two-way analysis of variance followed by Tukey–Kramer's *post-hoc* analysis. A value of $P<0.05$ was considered statistically significant.

DISCLOSURE

All the authors declared no competing interests.

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SUPPLEMENTARY MATERIAL

Table S1. Two-way ANOVA data of acetylcholine-induced relaxation of the aortic rings of WT and TG mice.

Figure S1. Representative immunofluorescent staining showing distribution of DDAH-1 in the aorta of mice.

Supplementary material is linked to the online version of the paper at http://www.nature.com/ki

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